Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biological Control 28 (2003) 92–100
Biological Control
www.elsevier.com/locate/ybcon

Soil as an environment for winter survival of aphid-pathogenic Entomophthorales

Charlotte Nielsen,[a,*] Ann E. Hajek,[b] Richard A. Humber,[c] José Bresciani,[a] and Jørgen Eilenberg[a]

[a] The Royal Veterinary and Agricultural University, Department of Ecology, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark
[b] Cornell University, Department of Entomology, Ithaca, NY 14853, USA
[c] USDA-ARS Plant Protection Research Unit, US Plant Soil and Nutrition Laboratory, Tower Road, Ithaca, NY 14853, USA

Received 6 August 2002; accepted 27 January 2003

Abstract

The survival of *Pandora neoaphidis* was studied for both discharged primary conidia and hyphal bodies inside aphid cadavers after storage on moist soil at different temperatures. The activity of the inoculum was quantified by the ability to produce replicate conidia as well as the ability to infect aphids. No effect of inoculum type was found. Conidia were produced after storage for at least 32 days at 20 °C, 64 days at 10 °C, and 96 days at 5 °C. Inoculum retained the ability to initiate infections in aphids after storage for at least 14 days at 20 °C, 32 days at 10 °C, and 64 days at 5 °C. Morphological studies of the inoculum suggest that *P. neoaphidis* may survive unfavorable conditions as thick-walled conidia also known as loricoconidia. Furthermore, *P. neoaphidis* and *Conidiobolus obscurus* were documented for the first time in field-collected soil in early spring by baiting the soil with aphids. We hypothesize that germination of overwintering inoculum is stimulated by host-induced factors since inoculum apparently responded to the presence of aphids.

Keywords: Aphids; Conidia; *Conidiobolus obscurus*; *Erynia neoaphidis*; Hyphal bodies; Loricoconidia; Overwintering; *Pandora neoaphidis*; Persistence; *Sitobion avenae*; Soil; Survival

1. Introduction

Pandora neoaphidis (Remaudière and Hennebert) Humber (Entomophthorales: Entomophthoraceae) (= *Erynia neoaphidis* Remaudière and Hennebert) is a well-known pathogen of many aphid species (Hemiptera: Aphidoidea). The fungus regularly causes epizootics among aphid pests and its prevalence may in some periods exceed 60% (Dean and Wilding, 1973; Feng et al., 1991; Steenberg and Eilenberg, 1995). This suggests great potential for utilizing *P. neoaphidis* for microbial control of aphids either by inoculation or by conservation of the environment. Regardless of the strategy, a better understanding of the epizootiology is essential for success. So far, most attention has been given to the effects of *P. neoaphidis* on aphid populations in economically important crops during the summer months, whereas knowledge concerning performance during the winter and initiation of infections in spring is limited.

In temperate regions, the majority of aphid pest species are holocyclic (Dixon, 1998), which means that their host insects are almost totally absent and thus makes the means for survival of *P. neoaphidis* and other aphid pathogens critical during winter periods. Unlike many species of entomophthoralean fungi, in vivo-produced resting spores (zygo- or azygospores) have never been observed for *P. neoaphidis* (Remaudière and Hennebert, 1980; Uziel and Kenneth, 1986).

In the literature, several hypotheses have been presented on the winter survival of *P. neoaphidis*. One hypothesis is that the fungus survives by continuous conidial infection in anholocyclic aphid populations and subsequently disperses to holocyclic aphids in the early summer (Byford and Reeve, 1969; Feng and Chen, 2002;

* Corresponding author. Fax: +45-35-28-26-70.
E-mail address: cni@kvl.dk (C. Nielsen).

1049-9644/$ - see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S1049-9644(03)00033-1

Wilding, 1973). This hypothesis is supported by the fact that *P. neoaphidis* is able to infect aphids at temperatures as low as 5 °C (Wilding, 1970). Furthermore, infections by *P. neoaphidis* have been found in populations of *Myzus persicae* Sulz. and *Rhopalosiphoninus staphyleae* (Koch) in Great Britain during April and May in beet clamps (Byford and Reeve, 1969) and on *M. persicae* overwintering on spinach in Arkansas (Elkassabany et al., 1992; McLeod et al., 1998). Finally, infections with *P. neoaphidis* have been observed in anholocyclic aphid populations in northern littoral France the whole year round even during winter (Remaudière et al., 1981).

There is strong evidence that *P. neoaphidis* has the ability to survive for longer periods outside its living host insect since soil is a natural source of inoculum of *P. neoaphidis* in autumn (Latteur, 1977). In addition, Latteur and Randall (1989) documented that primary conidia stored on the surface of soil produced replicate conidia for 6–8 months when incubated in constant dark at 5 °C. However, Morgan (1994) found that primary conidia stored on soil only produced replicate conidia for 1 month when incubated at 10 °C and for 24 days when incubated at 20 °C. It was subsequently concluded that, at least in Great Britain, the winter temperatures are not consistently low enough to ensure the survival of *P. neoaphidis* as conidia in the soil.

Finally, it has been suggested that *P. neoaphidis* survives the winter months as hyphal bodies inside aphid cadavers. It is well known that many entomophthoralean fungi, under cool and dry conditions survive for several months in dried cadavers (Courtois and Latteur, 1984; Latteur et al., 1985; Pell and Wilding, 1992; Tyrrell, 1988; Wilding, 1973). However, it seems that hyphal bodies of *P. neoaphidis* in mummified aphids are only able to survive in this stage for a few months under more humid conditions (Courtois and Latteur, 1984; Wilding, 1973). Under field conditions, Feng et al. (1992) observed that towards autumn, an increasing proportion of the aphids was filled with spherical hyphal bodies, which were clearly distinguishable from the regular hyphal bodies. These hyphal bodies inside aphid cadavers survived and infected aphids when placed on tree trunks during winter. Feng et al. (1992), consequently, proposed that the spherical hyphal bodies function as the morphological stage of winter survival. Such hyphal bodies have also been documented for another aphid pathogen, *Entomophthora planchoniana* Cornu, even though resting spores are known from this species (Keller, 1987a).

The objective of the present study was to clarify the mechanisms for winter survival of *P. neoaphidis*. The activity of two different inoculum types, namely discharged primary conidia and hyphal bodies inside aphid cadavers, was studied over time in the laboratory after storage on moist soil at several temperatures. The activity of inoculum was quantified as the ability to produce conidia as well as the ability to infect aphids. In addition, morphological studies of the stored inoculum were conducted and soil from the field was examined for naturally occurring aphid pathogens by baiting the soil with healthy aphids. For the latter study, soil was collected in early spring, before most aphid species had hatched from eggs.

2. Materials and methods

2.1. Laboratory bioassays

The soil used in the laboratory bioassays was collected from the surface (0–10 cm) of a winter wheat field near Ithaca, NY, USA. The soil was collected in December when soil moisture was high. To avoid natural-occurring entomopathogenic or saprophytic fungi, the soil was autoclaved at 121 °C for 90 min before use. 10 ± 2 g of moist soil (17% w/w) were placed in the bottom of a 35-mm plastic petri dish and subsequently smoothed and lightly compacted after which the dish was covered with a lid and sealed with parafilm.

The soil was inoculated with *P. neoaphidis* either with conidia or hyphal bodies. Inoculum originated from surface-sterilized cadavers of adult apterous *Sitobion avenae* F. previously infected by an in vitro culture of *P. neoaphidis* (ARSEF 5372 isolated from a Danish *Brevicoryne brassicae* L.) as described in Papierok and Hajek (1997). Contamination of the sterile soil was avoided by surface sterilization of all cadavers in 70% alcohol and 2% sodium hyphochlorite (Lacey and Brooks, 1997) before used in any experiment. The following procedures were used for inoculation of the soil. For the conidia treatment, three fresh sporulating cadavers were attached with vaseline to the lid of a petri dish which was then inverted over the soil. After 8 h of sporulation, a new sterile lid replaced the lid of the petri dish with cadavers attached. For the hyphal body treatment, two infected, but still not sporulating, aphids killed by *P. neoaphidis* were placed on the surface of the soil. The cadavers were kept in the petri dishes for the entire time of the experiment. The control consisted of petri dishes with soil, which were not subjected to any form of inoculum.

Immediately after inoculation the petri dishes were incubated in constant dark for 0, 4, 7, 14, 32, 64, and 96 days at three temperatures: 5, 10, and 20 °C. For each combination of treatment (2), temperature (3), and time (7), four petri dishes were evaluated (total 168 petri dishes) and for each combination of temperature and time one control petri dish was used (21 petri dishes). After storage, prior to testing the production of replicate conidia and infectivity of inoculum, the petri dishes were placed at 20 °C for 1 h in the dark to allow time for the shift in temperature.

The bioassays were performed by introducing eight newly molted apterous adult *S. avenae* obtained from a laboratory colony (originating from NY, USA) to each petri dish. In order to quantify the production of conidia, a coverslip (22×22 mm) was fastened to the lid with a drop of water. All petri dishes were inverted to ensure that ejected conidia landed on the coverslip. Aphids were then allowed to walk on the soil surface for 8 h in constant light at 20 °C and high humidity. After exposure, the aphids and the coverslip were removed from the petri dishes, and the aphids were incubated individually in 29-ml plastic cups containing 3% water agar and a fresh barley seedling (1–2 weeks old). All cups were incubated at 20 °C with a 14:10 h L:D photoperiod. The aphids were monitored on day 3 to day 6 after inoculation to detect infection with *P. neoaphidis*. Conidia adhering to the coverslip were counted under a microscope at 100× magnification for 24 equally distributed squares of 1.56 mm^2. In addition, conidia were collected from one of the petri dishes inoculated with conidia after storage at 10 °C for 96 days under a prolonged collection period of 48 h at 20 °C.

The effect of inoculum type, temperature, and length of storage on the percentage of infected aphids was analyzed by logistic regression. The GENMOD procedure in SAS v. 8.01 (SAS Institute, 1999) was used for the analysis, with inoculum type and temperature as class variables and with a binomial distribution and logit as link functions (Jensen and Skovgaard, 1995). Overdispersion was taken into account (Collet, 1991). The full model including all parameters and possible interactions was reduced stepwise by excluding the least significant parameter for each step but always by excluding interactions before main factors (Jensen and Skovgaard, 1995). The number of conidia ejected to the coverslips was analyzed by covariance analysis using the GLM procedure in SAS. Again, inoculum type and temperature were used as class variables but the data followed a normal distribution (Jensen and Skovgaard, 1995). The full model including all parameters and possible interactions was also reduced stepwise (Jensen and Skovgaard, 1995).

2.2. Morphological studies

To investigate the survival structures of *P. neoaphidis* in greater detail, non-sporulating *P. neoaphidis* cadavers of *S. avenae* were incubated in the dark on soil at 5 °C. After 1 month, cadavers were mounted in aceto-orcein (Humber, 1997) and examined with a light microscope at 400× magnification.

Furthermore, conidia produced in vitro from an egg yolk/sabouraud maltose agar plates (EYSMA) (Papierok and Hajek, 1997) and ejected onto the lid of a plastic petri dish under humid conditions were examined with both light and transmission electron microscope (TEM) after approximately 3 months of storage in constant dark at 17 °C. Conidia were scraped off the lid and fixed and embedded following the procedures described by Eilenberg et al. (1986). Thin sections of conidia were observed in a JEOL 1200 EX microscope at 60 kV.

2.3. Natural occurrence of aphid pathogens on soil

During 3 successive years, soil samples were taken before immigration of aphids to their summer hosts (personal observations) on the following dates: May 14 and June 3, 1997; March 17, 1998; and April 21 and May 19, 1999. Soil samples were taken at Zealand, Denmark from (1) organically grown agricultural fields which all had been grown with winter wheat during the previous season, (2) permanent grass, and (3) areas underneath bird cherry trees, *Prunus padus* L. (the primary host for *Rhopalosiphum padi* L). On each sampling date between 8 and 32 soil samples were taken from each habitat type from up to 4 localities per habitat type. The soil samples were collected from the upper 10-cm of the soil layer with a spoon without disturbing the soil and transferred to 3-cm petri dishes. The spoon was sterilized in 70% alcohol between each sample.

In the laboratory, petri dishes were incubated at 17 °C. During incubation the 24-h photoperiod was 12 h light in 1997 and 14 h in 1998 and 1999. Periodically, water was added to the petri dishes to keep the soil damp.

The soil was screened for the presence of Entomophthorales by repeated baiting of the soil surface with aphids. Aphids were obtained from a laboratory colony of *S. avenae* (originating from Denmark) maintained on barley seedlings. Each petri dish was baited with aphids four times during the experiment at the following time intervals: 0, 2, 7, and 14 days after sampling the soil.

Baiting was performed by allowing 12 3rd- to 4th-instar *S. avenae* nymphs to walk on the soil surface for 18 h. After exposure, the 12 aphids were removed from the soil and petri dishes with the soil samples were replaced in the incubator at 17 °C until the next assay began. The aphids removed from the petri dishes were incubated individually in a 30-ml plastic cup containing 3% water agar to maintain high humidity and a fresh barley seedling as food. All cups with the exposed aphids were incubated at 20 °C with a 16:8 h L:D photoperiod. The aphids were monitored daily for 1 week to detect infection with entomophthoralean fungi. Dead aphids were placed in humid chambers over microscope slides to allow conidial discharge. Conidia on the slides were mounted in lactic acid and entomophthoralean fungi were identified under the microscope (400×) based on morphological characters (Humber, 1989, 1997; Keller, 1987b, 1991).

The daily mean temperature, based on hourly recording, was measured 2 m above the soil surface at a

weather station in Højbakkegård, Zealand, Denmark, a locality situated near all sampling localities. For all years, day degrees (DD) from January 1 until baiting took place were calculated with a threshold temperature of 0 °C, since it is known that the arrival of *R. padi* and *S. avenae* into cereal fields can be expected 1100 DD and 1200 DD, respectively, after January 1, with a threshold temperature of 0 °C (Hansen, 1995).

The effects of year, DD, and habitat type were analyzed by logistic regression using the GENMOD procedure (SAS Institute, 1999). For each fungus species, separate analyses were performed. Year and habitat type were used as class variables and data were assumed to follow a binomial distribution.

3. Results

3.1. Laboratory bioassay

In the control treatment, none of the aphids died due to fungal infection, thus confirming that the soil used was free of aphid-pathogenic fungi. The control mortality was low and never exceeded 25% (2/8). In the fungal treatment, the percentage of mycosed individuals of *S. avenae* after exposure to inoculated soil is shown in Fig. 1. No effect of inoculum type on infection could be detected ($F_{1,161} = 1.07$; $P = 0.302$), and in petri dishes incubated with non-sporulating cadavers an intensive sporulation from cadavers were observed on day 4 and onwards at all incubation temperatures. Inoculum retained the ability to infect aphids for at least 14 days at 20 °C, 32 days at 10 °C, and 64 days at 5 °C. The number of mycosed individuals of *S. avenae* declined significantly with increasing storage time ($F_{1,162} = 322.04$; $P < 0.0001$) and increasing temperature ($F_{2,162} = 6.04$; $P = 0.0029$). The interaction between time and temperature was also significant ($F_{2,162} = 27.48$; $P < 0.0001$), indicating that the inoculum lost the ability to infect aphids at a greater overall rate at higher temperatures.

In agreement with the findings in the ability to infect aphids, no effect of inoculum type was found in the number of conidia produced ($F_{1,163} = 0.46$; $P = 0.496$) (Fig. 2). Conidia were produced for at least 32 days at 20 °C, 64 days at 10 °C, and 96 days at 5 °C. At all temperatures, the number of discharged conidia decreased with increasing time of storage ($F_{1,164} = 90.86$; $P < 0.0001$) and increasing temperature ($F_{2,164} = 7.48$; $P = 0.0008$).

In the additional experiment where conidia were collected from a prolonged period of 48 h at 20 °C from a dish inoculated with conidia and stored at 10 °C for 96 days, the number of replicate conidia reached the same number of conidia as obtained just after inoculation of the soil (data not shown).

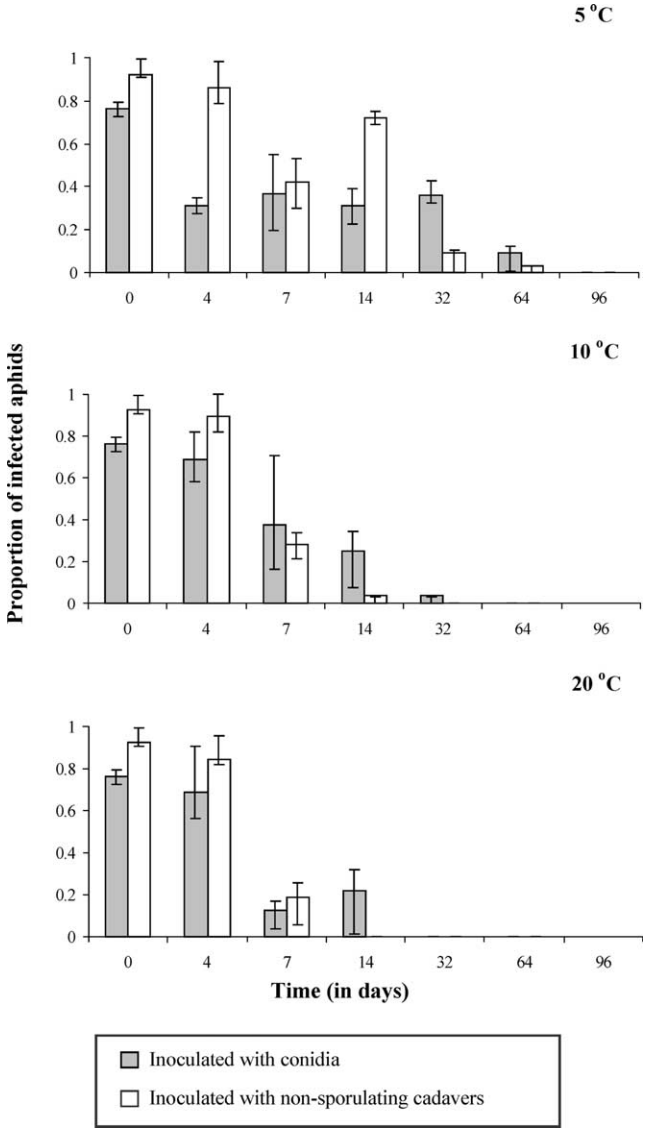

Fig. 1. Proportion of infected *Sitobion avenae* after subjection for 8 h to soil inoculated with either conidia (gray bars) or non-sporulating cadavers (white bars) and stored at 5, 10, or 20 °C. Error bars ± SE.

3.2. Morphological studies

In aphid cadavers stored on soil for a month, no unusual (spherical or thick-walled) hyphal bodies were observed. However, thick-walled conidia were observed externally on the cadavers. The shape and dimensions of these conidia corresponded well with conidial dimensions given for *P. neoaphidis* in Remaudière and Hennebert (1980). However, the walls of stored conidia were thickened and measured between 1.5 and 2.5 μm. The nuclei in these conidia were not stainable with aceto-orcein, but when the conidial wall was broken by physical pressure, one small nucleus was observed in each conidia after staining. Similar thick-walled conidia were also observed for in vitro-produced conidia when

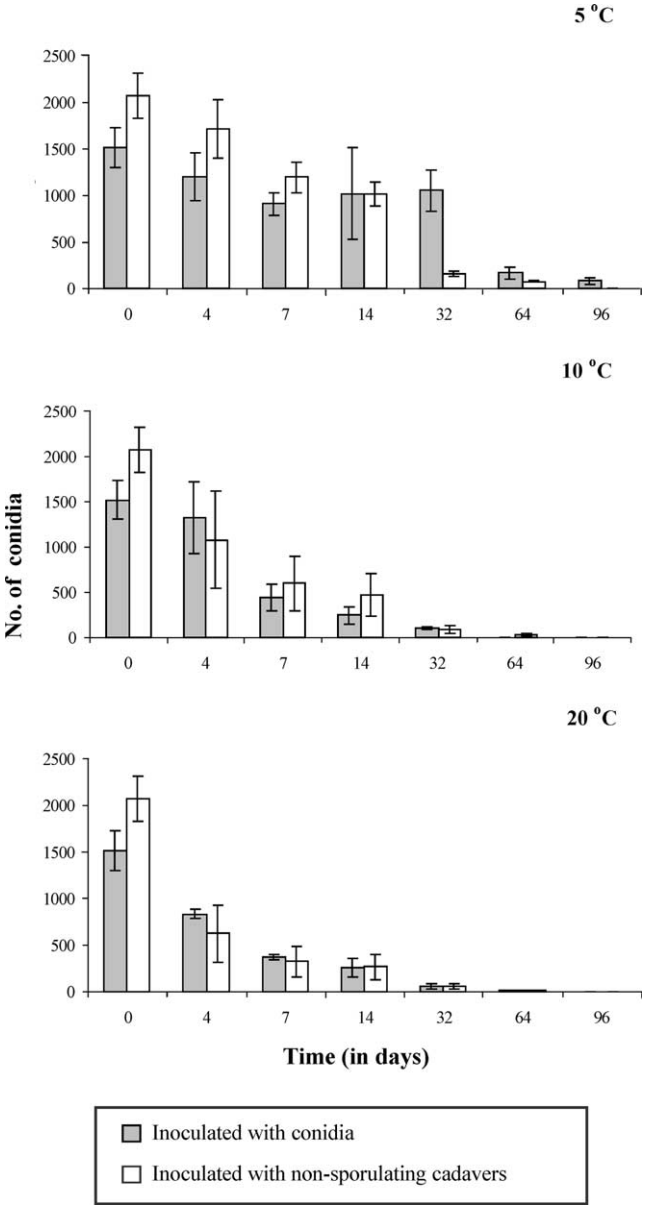

Fig. 2. Production of replicate conidia per 37.44 mm^2 during 8 h incubation at 20 °C. Prior to the test, the soil was inoculated either with conidia (white bars) or non-sporulating cadavers (hatched bars) and stored at 5, 10, or 20 °C. Error bars ± SE.

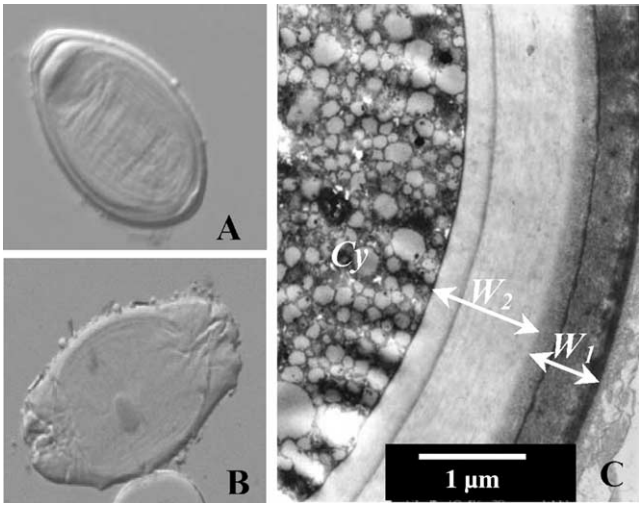

Fig. 3. (A) Light micrographs of a thick-walled conidium (in vitro) fixed in aceto-orcein. (B) Light micrographs of a thick-walled conidium (in vitro) after destruction of the wall by physical pressure. A condensed nucleus is seen in the center. (C) Transmission electron micrograph of a thick-walled conidium produced in vitro. Cy, cytoplasm; W_1, electron dense outer wall layers (approximately 700 nm); W_2, electron translucent wall layers (approximately 1000 nm).

stored in cold without nutrients in constant dark under humid conditions (Fig. 3). TEM pictures of the in vitro-produced thick-walled conidia showed that the wall consisted of several distinct layers with an internal structure of granular appearance (Fig. 3C).

3.3. Natural occurrence of aphid pathogens on soil

Bioassay results showed that *S. avenae* allowed to walk on soil collected in Denmark in spring became infected with both *P. neoaphidis* and *Conidiobolus obscurus* (Hall and Dunn) Remaudière and Keller during all 3 years of this study. The percentage of soil samples (data from all habitats and localities are pooled) containing active inoculum as evidenced by fungal infection of *S. avenae* is shown as a function of days after sampling in Table 1. A maximum of 28% (11/40) of the petri dishes contained active inoculum of *P. neoaphidis* at a given time (April 1999). For *C. obscurus* the highest level of infection was obtained in April and May 1999 where 15% (6/40) of the petri dishes contained active inoculum.

The presence of active inoculum was not correlated with the year of testing or with DD until testing for either *P. neoaphidis* or *C. obscurus* (Year: *P. neoaphidis* ($F_{2,10} = 2.63$; $P = 0.121$); *C. obscurus* ($F_{2,10} = 0.38$; $P = 0.695$)) (DD: *P. neoaphidis* ($F_{1,16} = 0.11$; $P = 0.749$); *C. obscurus* ($F_{1,16} = 0.09$; $P = 0.770$)). In Fig. 4 the percentage of soil samples containing active inoculum is shown as a function of the three habitat types from which soil was sampled (data from all years pooled). Soil samples collected from agricultural fields had a consistently higher presence of active inoculum of *P. neoaphidis* $F_{2,12} = 3.06$; $P = 0.047$). For *C. obscurus*, habitat type was not associated with inoculum presence ($F_{2,12} = 0.96$; $P = 0.410$).

4. Discussion

Our study demonstrated that *P. neoaphidis* inoculum retains the ability to produce replicate conidia for several months at low temperatures and was the first to prove that inoculum of *P. neoaphidis* stored for several months actually can infect aphids. Furthermore, no

Table 1
The presence of entomophthoralean fungi on soil surfaces documented by infection of *Sitobion avenae* after contact with the soil. Percent soil samples which caused infection with entomophthoralean fungi are shown as a function of incubation time at 17 °C and 12:12 L:D in 1997 and 14:10 L:D in 1998 and 1999. For each baiting time the time is also expressed as day degrees (DD) from 1 January, with a threshold temperature of 0 °C

Fungus	Date and year	No. of soil samples tested	0 days after sampling	2 days after sampling	7 days after sampling	14 days after sampling
P. neoaphidis	May 14, 1997	40	0% (525 DD)	0% (559 DD)	5% (644 DD)	10% (763 DD)
	June 3, 1997	48	0% (723 DD)	0% (757 DD)	4% (842 DD)	4% (961 DD)
	March 17, 1998	40	0% (271 DD)	—	5% (390 DD)	0% (509 DD)
	April 21, 1999	40	0% (368 DD)	0% (402 DD)	28% (487 DD)	13% (606 DD)
	May 19, 1999	40	0% (626 DD)	0% (660 DD)	15% (725 DD)	—
C. obscurus	May 14, 1997	40	0% (525 DD)	0% (559 DD)	0% (644 DD)	8% (763 DD)
	June 3, 1997	48	0% (723 DD)	0% (757 DD)	0% (842 DD)	8% (961 DD)
	March 17, 1998	40	0% (271 DD)	—	13% (390 DD)	10% (509 DD)
	April 21, 1999	40	0% (368 DD)	3% (402 DD)	5% (487 DD)	15% (606 DD)
	May 19, 1999	40	0% (626 DD)	0% (660 DD)	15% (725 DD)	—

difference in the ability to produce conidia or to infect aphids was found between hyphal bodies or conidia stored on soil. At 10 and 20 °C, our results on production of conidia are comparable with earlier findings by Perry et al. (1982), Latteur and Randall (1989), and Morgan (1994). However, at 5 °C we found very few conidia produced after 3 months of storage in comparison to the earlier reports (Latteur and Randall, 1989; Morgan, 1994; Perry et al., 1982). We only collected conidia for 8 h beginning from 1 h after moving samples to 20 °C. Contrary to our study, Morgan (1994) collected conidia for 24 h. Perry et al. (1982) and Latteur and Randall (1989) did not give the exact time over which conidia were collected but in both cases, it was for more than 1 day. To investigate the impact of conidial collection time, we collected conidia over an additional 48 h after storage at 10 °C for 3 months. We found that the prolonged collection time gave the same number of conidia as obtained before cold storage. This indicates that the length of time for conidia collection probably is a significant factor for the production of replicate conidia when primary conidia have been stored under cold conditions.

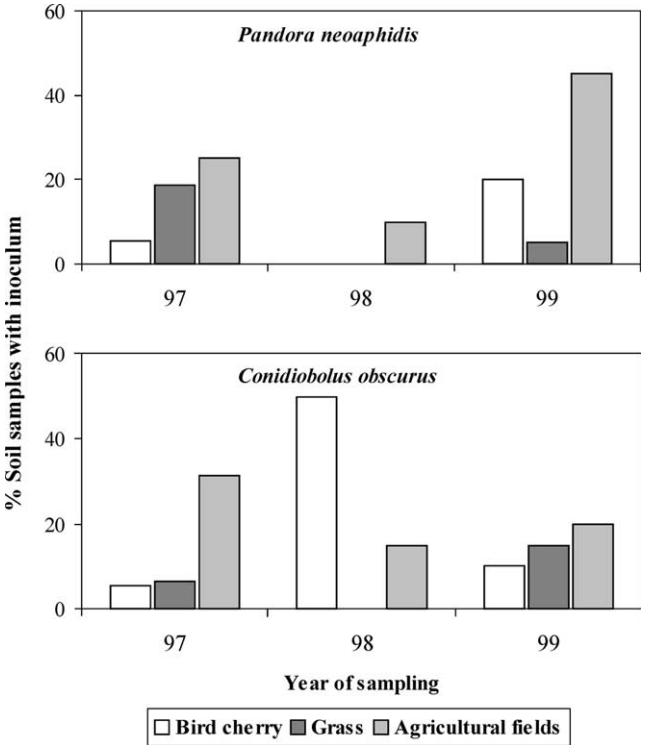

Fig. 4. Entomophthoralean fungi on soil surface documented by infection of *Sitobion avenae* after contact with the soil. Percent soil samples which caused infection by the entomophthoralean fungi *Pandora neoaphidis* and *Conidiobolus obscurus* are shown as a function of habitat type.

In our morphological studies of stored inoculum of *P. neoaphidis*, thick-walled conidia appeared. The thick-walled conidia had non-staining nuclei, presumably because of failure of the stain to penetrate the wall. Furthermore, these conidia had granular cytoplasm and a relatively thick wall, which is similar to immature resting spores of other entomophthoralean fungi (Hajek and Humber, 1997; Latgé et al., 1982). Such thick-walled conidia were first described by Weiser and Batko (1966) from *Entomophthora destruens* Weiser and Batko [= *Conidiobolus destruens* (Weiser and Batko)

Ben-Ze'ev and Kenneth]. They referred to these structures as loricoconidia or resting spores formed from conidia. Based on our morphological studies of stored *P. neoaphidis* inoculum we suggest, that loricoconidia may function as one overwintering form in the life cycle of *P. neoaphidis*, especially under humid conditions such as the soil environment. However, further studies of these thick-walled conidia are needed, especially on their germination.

Entomophthoralean fungi may have more than one possible means of survival available. For example, *P. neoaphidis* may survive as hyphal bodies under relatively dry conditions (Feng et al., 1992) or as loricoconidia under more humid conditions as we propose here. Different mechanisms of overwintering is also known from another aphid pathogen, *E. planchoniana*, since overwintering for this species may take place as either hyphal bodies on tree trunks or as resting spores in the soil (Keller, 1987a,b).

Active inoculum of *P. neoaphidis* and *C. obscurus* was documented for the first time in field-collected soil in early spring although a winter had passed since aphids were present on plants above the soil. The two fungi were found in both undisturbed habitats and agricultural fields but most frequently in the latter. This trend was seen even though some of the soil samples were taken after plowing the soil. This might be attributed to the density of aphids and, subsequently, the density of infected aphids often reaches much higher levels in agricultural fields than in mixed habitats. We only know of one other study (Latteur, 1977) where soil has been baited with aphids and, like our study, both *P. neoaphidis* and *C. obscurus* were present. However, all of Latteur's (1977) soil samples were taken in autumn from Belgian vetch fields relatively shortly after *P. neoaphidis* and *C. obscurus* epizootics had occurred in populations of *Acyrthosiphon pisum* Harris. Therefore, it could not be stated whether the inoculum was actually able to survive the winter. In our study and that by Latteur (1977), other common aphid pathogens such as *E. planchoniana* or *Neozygites fresenii* (Nowakowski) Remaudière and Keller were not found.

The breaking of dormancy or quiescence is thought to be associated with a range of factors including the fungal species and isolate as well as temperature, soil moisture, location in the soil strata and day length (Hajek, 1997; Hajek and Humber, 1997). Moreover, aromatic compounds have been documented as playing an important role in the germination of resting spores of the aphid pathogen *Conidiobolus thromboides* Drechsler (Soper et al., 1975). Whether aromatic compounds derived from the host insect can activate or stimulate the germination of survival structures has never been elucidated. However, Hajek and Eastburn (2001) did not find any host-induced factors associated with germination of the gypsy moth pathogen *Entomophaga maimaiga* Humber, Shimazu and Soper. Nevertheless, it seems that the timing of resting spore germination is well synchronized with the seasonality of the gypsy moth (Hajek, 1997; Hajek and Humber, 1997).

In our study, activity of natural inoculum was measured by repeated baiting of soil samples with aphids. The bait aphids were never infected when exposed to soil samples coming directly from the field and it is, therefore, likely that the soil-borne inoculum is dormant or quiescent in spring. However, by repeated baiting of the same soil samples, the earliest that *P. neoaphidis* infections were detected was in the cohort of aphids exposed to the soil 7 days after the soil had been collected and the first baiting with aphids took place. For *C. obscurus*, infections were only seen in the cohort of aphids exposed to the soil on day 2, 7, and 14 after sampling and never in cohort of aphids exposed to soil 0 days after sampling. The activity of inoculum was not correlated with DD even though it is known that for cereal aphids, both egg hatch and immigration to cereal fields are controlled by day-degrees. We could detect *P. neoaphidis* and *C. obscurus* a long time before the number of DD required for immigration of *S. avenae* and *R. padi* was attained but never until several days after the first contact with bait aphids. We hypothesize that germination of overwintering inoculum is stimulated by host-induced factors, since it seems that the quiescent inoculum became activated only in the presence of aphids. This was supported in 1998 when soil was sampled from a lucerne field in late March after a very mild winter. Bait aphids allowed to walk on this soil were infected with both *P. neoaphidis* and *C. obscurus* just after sampling the soil (unpubl. data). In Denmark, pea aphids, *A. pisum*, overwinter as living individuals on peas and lucerne during mild winters (Nielsen and Jensen, 1994). Unfortunately the exact lucerne field from that study was not checked for the presence of overwintering aphids in 1998.

In summary, we conclude that the fungal inoculum present in the soil in spring is an important factor for initiation of infections in aphid populations in spring. Furthermore, several authors have reported that a very high proportion (up to 50%) of cereal aphids fall to the ground each day (Griffiths et al., 1985; Sopp et al., 1987; Sunderland et al., 1986), especially during spring (Sopp et al., 1987). Occurrence of aphids on the soil would, therefore, result in infection as we found.

Acknowledgments

We thank Stewart M. Gray and Dawn M. Smith for assistance and instruction in rearing the aphids. The Danish Environmental Research Programme and The Danish Environmental Protection Agency provided financial support.

References

Byford, W.J., Reeve, G.J., 1969. *Entomophthora* species attacking aphids in England, 1962–1966. Trans. Br. Mycol. Soc. 52, 342–346.

Collet, D., 1991. Modelling Binary Data. Chapman & Hall, London.

Courtois, P., Latteur, G., 1984. Étude quantitative de la survie des corps hyphaux d'*Erynia neoaphidis* Remaud. et Henn. (Zygomycetes Entomophthoraceae) en fonction de la température et de l'humidité relative. Parasitica 40, 211–220.

Dean, G.J.W., Wilding, N., 1973. Infection of cereal aphid by the fungus *Entomophthora*. Ann. Appl. Biol. 74, 133–138.

Dixon, A.F.G., 1998. Aphid Ecology: An Optimization Approach. Chapman & Hall, London.

Eilenberg, J., Bresciani, J., Latgé, J.P., 1986. Ultrastructural studies of primary spore formation and discharge in the genus *Entomophthora*. J. Invertebr. Pathol. 48, 318–324.

Elkassabany, N.M., Steinkraus, D.C., McLeod, P.J., Correll, J.C, Morelock, T.E., 1992. *Pandora neoaphidis* (Entomophthorales: Entomophthoraceae): a potential biological control agent against *Myzus persicae* (Homoptera: Aphididae) on spinach. J. Kansas Entomol. Soc. 65, 196–199.

Feng, M.G., Chen, C., 2002. Incidence of infected *Myzus persicae* alatae trapped in fligth imply place-to-place dissemination of entomophthoralean fungi in aphid populations through migration. J. Invertebr. Pathol. 81, 53–56.

Feng, M.G., Johnson, J.B., Halbert, S.E., 1991. Natural control of cereal aphids (Homoptera: Aphididae) by entomopathogenic fungi (Zygomycetes: Entomophthorales) and parasitoids (Hymenoptera: Braconidae and Encyrtidae) on irrigated spring wheat in southwestern Idaho. Environ. Entomol. 20, 1699–1710.

Feng, M.G., Nowierski, R.M., Klein, R.E., Scharen, A.L., Sands, D.C., 1992. Spherical hyphal bodies of *Pandora neoaphidis* (Remaudière & Hennebert) Humber (Zygomycetes: Entomophthorales) on *Acyrthosiphon pisum* (Harris) (Homoptera: Aphididae): a potential overwintering form. Pan-Pacific Entomol. 68, 100–104.

Griffiths, E., Wratten, S.D., Wickerman, G.P., 1985. Foraging by the carabid *Agonum dorsale* in the field. Ecol. Entomol. 10, 181–189.

Hajek, A.E., 1997. Ecology of terrestrial fungal entomopathogens. Adv. Microbial Ecol. 15, 193–249.

Hajek, A.E., Eastburn, C.C., 2001. Effect of host insects on activation of *Entomophaga maimaiga* resting spores. J. Invertebr. Pathol. 77, 290–291.

Hajek, A.E., Humber, R.A., 1997. Formation and germination of *Entomophaga maimaiga* azygospores. Can. J. Bot. 75, 1739–1747.

Hansen, L.M., 1995. Bladlus—det nationale skadedyr i Danmark. SP Rapport 4, 115–128, (in Danish).

Humber, R.A., 1989. Synopsis of a revised classification for Entomophthorales (Zygomycotina). Mycotaxon 34, 441–460.

Humber, R.A., 1997. Fungi: identification. In: Lacey, L. (Ed.), Manual of Techniques in Insect Pathology. Academic Press, San Diego, CA, pp. 153–186.

Jensen, J.E., Skovgaard, I., 1995. Introduction til SAS med statistiske anvendelser. Jordbrugsforlaget, Copenhagen, Denmark (in Danish).

Keller, S., 1987a. Observation on the overwintering of *Entomophthora planchoniana*. J. Invertebr. Pathol. 50, 333–335.

Keller, S., 1987b. Arthropod-pathogenic Entomophthorales of Switzerland.I. *Conidiobolus*, *Entomophaga* and *Entomophthora*. Sydowia 40, 122–167.

Keller, S., 1991. Arthropod-pathogenic Entomophthorales of Switzerland. II. *Erynia*, *Eryniopsis*, *Neozygites*, *Zoophthora*, and *Tarichium*. Sydowia 43, 39–122.

Lacey, L.A., Brooks, W.M., 1997. Initial handling and diagnosis of diseased insects. In: Lacey, L. (Ed.), Manual of Techniques in Insect Pathology. Academic Press, San Diego, CA, pp. 1–15.

Latgé, J.P., Prévost, M.C., Perry, D.F., Reisinger, O., 1982. Étude en microscopie électronique de *Conidiobolus obscurus*. I. Formation et germination des azygospores. Can. J. Bot. 60, 413–431.

Latteur, G., 1977. Sur la possibilité d'infection directe d'Aphides par *Entomophthora* à partir de sols hérbergeant un inoculum naturel. Comptes Rendus Acad. Sci. Paris 284 (D), 2253–2256.

Latteur, G., Randall, L., 1989. The survival of inoculum of conidia of *Erynia neoaphidis* Remaud. and Henn. on unsterilized soil. IOBC/WPRS Bulletin X11/4, p. 72.

Latteur, G., Lizen, E., Oger, R., 1985. Influence de divers facteurs physiques sur la virulence des conidies de l'Entomophthorale *Erynia neoaphidis* Remaud. et Henn. envers le puceron du pois, *Acyrthosiphon pisum* (Harris). Parasitica 41, 151–162.

McLeod, P.J., Steinkraus, D.C., Correll, J.C., Morelock, T.E., 1998. Prevalence of *Erynia neoaphidis* (Entomophthorales : Entomophthoraceae) infections of green peach aphid (Homoptera : Aphididae) on spinach in the Arkansas River Valley. Environ. Entomol. 27, 796–800.

Morgan, L.W., 1994. Survival, germination responses and infectivity of conidia of *Erynia neoaphidis* (Zygomycetes: Entomophthorales). Ph.D. Thesis, University of Wales, Cardiff, UK.

Nielsen, G.C., Jensen, J.P., 1994. "Markens Sygdomme og Skadedyr". Dalum Landbrugsskoles Forlag, Denmark (in Danish).

Papierok, B., Hajek, A.E., 1997. Fungi: Entomophthorales. In: Lacey, L. (Ed.), Manual of Techniques in Insect Pathology. Academic Press, San Diego, CA, pp. 187–212.

Pell, J.K., Wilding, N., 1992. The survival of *Zoophthora radicans* (Zygomycetes: Entomophthorales) isolates as hyphal bodies in mummified larvae of *Plutella xylostella* (Lep.: Yponomeutidae). Entomophaga 37, 649–654.

Perry, D.F., Latteur, G., Wilding, N., 1982. The environmental persistence of propagules of the Entomophthorales. In: Proc. Intern. Colloq Insect Pathol. University of Sussex, Brighton, pp. 325–330.

Remaudière, G., Hennebert, G.L., 1980. Révision systématique des *Entomophthora aphidis* Hoffm. in Fres. Description de deux nouveaux pathogènes d'aphides. Mycotaxon 11, 269–321.

Remaudière, G., Latgé, J.P., Michel, M.F., 1981. Ecologie comparée des Entomophthorales pathogènes de puceron en France Littorale et Continental. Entomophaga 26, 157–178.

SAS Institute Inc., 1999. SAS/STAT User's Guide, Version 8, First edition, vol. 2. SAS Institute Inc., Cary, NC, USA.

Soper, R.S., Holbrook, F.R., Majchrowicz, I., Gordon, C.C., 1975. Production of *Entomophthora* resting spores for biological control of aphids. Life Sci. Agric. Exp. Stn. Tech. Bull. 75, Univ. Maine Orono, 15pp.

Sopp, P.I., Sunderland, K.D., Coombes, D.S., 1987. Observations on the number of cereal aphids on the soil in relation to aphid density in winter wheat. Ann. Appl. Biol. 111, 53–57.

Steenberg, T., Eilenberg, J., 1995. Natural occurrence of entomopathogenic fungi on aphids at an agricultural field site. Czech. Mycol. 48, 89–96.

Sunderland, K.D., Fraser, A.M., Dixon, A.F.G., 1986. Field and laboratory studies on money spiders (Linyphiidae) as predator of cereal aphids. J. Appl. Ecol. 23, 433–447.

Tyrrell, D., 1988. Survival of *Entomophaga aulicae* in dried insect larvae. J. Invertebr. Pathol. 52, 187–188.

Uziel, A., Kenneth, R.G., 1986. In vitro resting spore formation in *Erynia neoaphidis*. In: Samson, R.A., Vlak J.M., Peters, D. (Eds.), Fundamental and Applied Aspects of Invertebrate Pathology. Proc. Intern. Colloq Insect Pathol.,Veldhoven, The Nederlands, p. 230.

Weiser, J., Batko, A., 1966. A new parasite of *Culex pipiens* L., *Entomophthora destruens* sp. nov. (*Phycomycetes*, *Entomophthoraceae*). Folia Parasitol. (Praha) 13, 144–149.

Wilding, N., 1970. The effect of temperature on the infectivity and incubation period of the fungi *Entomophthora aphidis* and *E. thaxteriana* on the pea aphid, *Acyrthosiphon pisum*. Proc. Intern. Colloq Insect Pathol., College Park, MD, pp. 84–88.

Wilding, N., 1973. The survival of *Entomophthora* spp. in mummified aphids at different temperatures and humidities. J. Invertebr. Pathol. 21, 309–311.